PROTOCOL

for

COMMERCIAL MICROBIOLOGICAL AMENDMENT TESTING AND EVALUATION

to

Air Force Center for Environmental Excellence
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by

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1.0 INTRODUCTION

The Air Force has identified numerous sites contaminated with petroleum hydrocarbons and is currently involved in an extensive effort to clean up many of these sites. Physical technologies such as soil vacuum extraction and biological technologies such as bioventing have been used successfully to accomplish site closure. Although these technologies are applicable at many sites, there may be sites that can be more readily treated using certain amendments.

A typical amendment product consists of one or any combination of components including a bacterial preparation, a nutrient stock, buffers for pH control, and/or surfactants. Most often, the constituents of the amendments are purchased in a dried state and must be hydrated prior to application. Amendments are added through surface application, by mixing them with excavated soils, or by direct injection into subsurface soils. Application rates are both amendment and site specific and usually are specified by the vendor.

This protocol was developed for the Air Force to use as a tool for evaluating commercially available amendments by determining any enhancement in remediation of petroleum hydrocarbon contamination under controlled conditions. The protocol consists of a series of tests designed to determine any enhancements attributable to the addition of a microbial component or chemical components. The tests involve examining the soil characteristics, the microbial amendment, and the fate of the contaminant.

Certain soil characteristics including moisture, pH, organic matter content, alkalinity, and particle size distribution are important parameters that affect microbial activity and should be measured on all soils that are candidates for amendment treatment.

Microbial activity is strongly influenced by the availability of nutrients, and in soils the supply of necessary nutrients can be low thus limiting contaminant degradation. The more critical nutrients typically are in short supply are nitrogen, phosphorous, and potassium. To properly evaluate microbial amendments, it is necessary to measure the concentrations of these nutrients in the soil both with and without amendment addition. It is also important to measure nutrient concentrations at the end of incubation to determine if the supply of available nutrients became limited during the test.

A number of methods are available for monitoring microbial activity, including respirometry and enumeration. Respirometry is an indirect method that measures microbial activity based on oxygen utilization and carbon dioxide production. Because hydrocarbon degradation is an aerobic process, the oxygen utilization rate can be used to estimate the hydrocarbon degradation rate.

Enumerating microorganisms that can degrade target contaminants is a valuable tool for determining the contribution of microbes with the desired capability that a specific microbial amendment provides to soils. It is important to conduct the enumeration analysis to determine the number of hydrocarbon-degrading bacteria in the inoculum to quantify the number of these organisms that are added to the soil during inoculation.

The most important parameter used to compare the effectiveness of an amendment against indigenous microbial activity is the reduction in contaminant. It is necessary to measure the hydrocarbon content of the soil before and after treatment and in the gas exchanged during reactor operation. If an amendment is to be deemed effective, the hydrocarbon reduction in the amended soil must significantly exceed the reduction in the unamended soil.

The following sections contain detailed descriptions of the preparation, experimental setup, monitoring activities, and analytical methods to be conducted to perform an evaluation under controlled conditions. These methods have been selected based on the results of extensive testing and analyses during protocol development experiments. The suite of analyses will provide the information necessary to determine if the use of the amendment would provide any significant enhanced remediation capacity over that of indigenous microorganisms.

2.0 OBJECTIVE

The objective of this protocol is to provide a standardized testing and evaluation procedure that can be used by the Air Force to screen commercially available microbial amendment products. The objective of testing and evaluating commercial microbial amendments is to demonstrate, under carefully controlled conditions, whether these amendments provide a significant improvement over indigenous microorganisms in the biodegradation of petroleum hydrocarbons.

3.0 EXPERIMENTAL METHODS

3.1 Reactor Design

The tests are conducted using a reactor flask design. Each reactor consists of a 500-mL wide-mouth Erlenmeyer flask fitted with a Neoprene™ rubber stopper. Two 18-gauge stainless steel Luer lock needles are inserted through the rubber stopper to facilitate sampling and exchanging reactor atmospheres.

One of the needles is 6 inches long and is placed so that the tip is below the soil surface and just above the bottom of the flask. This needle serves as the influent line for introducing air during atmospheric exchanges. The second needle is approximately 2 inches long and is placed so that the tip is just below the bottom of the rubber stopper. This needle is used to extract gas during sampling and atmospheric exchanging.

The head of each needle is equipped with a two-way Luer lock valve to provide a mechanism for both removing gas and sealing the reactors. The valves are designed for syringe attachment. This design allows gas exchange to closely simulate conditions seen in bioventing and/or biopile configurations.

3.2 Experimental Conditions

Three experimental conditions are established to evaluate the performance of the amendment product. Triplicate reactors are set up for each condition using a common batch of contaminated soil. The conditions examined include contaminated soil without any amendment, contaminated soil with a filter-sterilized amendment preparation, and contaminated soil with an amendment added according to the vendor's specifications.

The contaminated soil without amendment provides the baseline degradation data against which the amendment is compared. Because nothing is added to the soil under this condition, any degradation is the result of indigenous microorganisms. The results from this condition are representative of the degradation that might be obtained during treatment without amendment addition.

The contaminated soil with the sterilized amendment addition provides data on any contribution from the nonbiological components of the amendment product to the biodegradative capacity of the indigenous microorganisms. Added nutrients could provide these microorganisms with a needed supply to support a higher rate of activity than is seen under the nonamended condition. Surfactants often are a component of amendment products, and their addition could increase the bioavailability of

certain contaminant compounds and increase their biodegradation rate. The data from this condition are compared against the data from the nonamended condition to determine if there are enhancements.

The final condition is set up with contaminated soil and the amendment product added according to the vendor's specifications. The preparation procedure for the product, the application rate(s), and any special application directions must be obtained from the vendor. It is critical that vendors provide this information to ensure that the products are properly evaluated. Vendors that do not provide clear and concise directions stand the chance of having their products fail the evaluation, and the argument that a product was not evaluated properly cannot be accepted.

3.3 Soil Processing and Amendment Addition

The procedures for soil processing and amendment addition are described in the following paragraphs.

Prior to preparing the soils, weigh and label three porcelain crucibles as described in Section 4.7 below. Label one large Tedlar™ soil sampling bag and three smaller bags. Prepare all materials, supplies, and media for all of the analytical methods described in Section 4.0, accordingly. Once these preparations are complete, prepare the soil as follows.

Prepare the soil in two steps. First, transfer an amount of soil sufficient to accommodate the number of reactors under all three conditions into the large Tedlar™ soil sampling bag. Seal the bag and hand-knead until the contents appear homogeneous. This step is extremely important because the soil will be segregated into the stocks for the three conditions. Once the soil appears homogeneous, remove an aliquot, analyze it by gas chromatography (GC) (see Section 4.1), and record the TPH value. During the analysis, continue to knead the bag and pull a second sample for TPH analysis. Repeat the kneading process for a third time, and again measure the TPH in the soil. This process is repeated until the coefficient of variance (CV) of three consecutive TPH values is 10% or less. Once this level of homogeneity is achieved, the soil is ready for the next step.

Following the kneading process described above, weigh triplicate 10-g samples into each of the three preweighed and labeled porcelain crucibles described above and conduct a baseline moisture content analysis of the homogenized soil (see Section 4.7). Once the moisture content is determined, the soil can be segregated into the three smaller Tedlar™ bags to be readied for the three experimental conditions. Divide the homogenized soil into three separate stocks, making sure to transfer enough soil to meet the requirements for each condition.

Prepare the amendment according to the vendor's guidelines. The amendment dosage and moisture content are determined based on the vendor's specifications for the particular microbiological amendment product. If the vendor does not provide moisture content recommendations, prepare the amendment to achieve a final moisture content of 20%. Once the amendment is prepared, divide the stock into two equal parts.

Sterilize one part of the amendment by filtering it through a 0.22-μm membrane filter into a sterilized receiver. This stock will be used to prepare the contaminated soil with sterilized amendment condition. In addition, filter-sterilize an equal volume of distilled (or equivalent grade) water to use for moisture adjustment in the nonamended condition.

Add the required amount of the appropriately prepared amendment to the volume of soil in the bags for each respective condition. Add the volume of sterilized water necessary to achieve the targeted moisture content to the soil in the bag not receiving amendment. Seal the bags, taking care to minimize headspace.

Thoroughly hand-knead the soils in the bags until the contents are well mixed (up to 1 hour). Transfer an appropriate volume of each soil into clean, prelabeled containers to conduct the initial soil analyses listed in Section 4.0.

3.4 Reactor Setup

Label a reactor flask with the amendment identification (ID), the experimental condition, the replicate number, the date of setup, and the initials of the preparer. Tare the flask on a two-place balance. Transfer enough of the appropriate homogenized soil into the reactor to fill the flask to the 200-mL mark and record the soil weight. Continue this procedure and fill the remaining reactors. Place the stopper with the needles in place into the mouth of each flask. Make sure that the longer needle is situated just above the bottom of the flask. Insert the stopper so that a tight seal is established and check to make sure that the valves on each flask are closed. Place the reactors in an upright position in a temperature-controlled incubator maintained at 25°C unless an alternative temperature is specified by the vendor and/or the Air Force.

3.5 Reactor Operation

3.5.1 Maintaining Temperature. The temperature on the incubator is checked on a regular basis to ensure that the reactors are kept at the desired temperature. In the event that the temperature varies by more that a few degrees, the incubator is readjusted to the appropriate setting.

3.5.2 Atmosphere Exchanges. To maintain an aerobic environment in each reactor, it is necessary to regularly exchange the reactor atmospheres. The exchanges are made approximately once per week; however, the required exchange rate must be determined by measuring the oxygen concentrations in the reactor headspace (see Section 4.3). The atmospheres are exchanged when the oxygen concentrations drop below 10%.

The atmospheres are exchanged using a direct-flush method. Fill a 1-L syringe with 300 mL of clean air and connect the syringe to the valve on the long needle. Connect a 500-mL Tedlar™ gas sampling bag to the valve on the shorter needle. Open the valve on the Tedlar™ bag, then open the valve on the shorter needle. Next, open the valve on the longer needle and slowly push the 300 mL of air from the syringe through the rector. As the air is forced from the syringe, the Tedlar™ bag will inflate. Allow the reactor to come to equilibrium at ambient pressure. Close all of the valves on the reactor and the valve on the Tedlar™ bag. Remove the syringe and the bag. The gas in the bag is ready for analysis.

3.6 Process Monitoring

Oxygen and carbon dioxide concentrations are measured in the gas collected from each reactor during atmosphere exchanges (see Section 4.3). The data are used to monitor the oxygen concentrations to ensure that oxygen levels do not become rate-limiting and to estimate biodegradation rates. The final evaluation of biodegradation performance of an amendment under each of the experimental conditions is assessed using TPH data from analyses of initial and final soil samples (see Section 4.1).

The parameters listed in Table 1 are measured in the initial soil samples collected from the homogenized batches of soil at the time of reactor setup. The data are used as the baseline against which to evaluate the biodegradation in each of the reactors. The final soil samples are collected from each reactor after 30 days of incubation. These samples are analyzed for the parameters listed in Table 1. The baseline data are subtracted out, and the resulting data for each experimental condition are compared.

3.7 Reactor Harvesting

After the 30-day incubation period, the reactors are harvested and the soil is removed for analysis. Each reactor is harvested individually to maintain the integrity of the sample and to minimize the potential for cross-contamination between reactors.

Table 1. Sampling Schedule for Testing and Evaluating Commercial Microbiological Amendments.

Analysis	Initial	Intermediate	Final
Soil pH	S		S
Soil Moisture	S		S
Alkalinity	S		S
Particle Size Distribution	S		
Textural Analysis	S		
Cation Exchange Capacity	S		
Organic Matter Content	S		S
Nutrients (N,P,&K)	S		S
Microbial Enumerations	S,I		
Oxygen and Carbon Dioxide	G	G	G
Hydrocarbon in Gas	G	G	G
Hydrocarbon in Soil	S		S

S = Soil Samples
I = Inoculum
G = Gas Samples

Before removing the soil from a reactor, lay out a piece of clean nonabsorbent or wax paper to contain the soil sample if it is spilled. The paper should be discarded and replaced with a clean piece after harvesting each reactor. Prepare all materials, supplies, and media needed for all of the analyses to be conducted on the soils collected during harvesting. Label all transfer bottles, tubes, and vials appropriately to facilitate the harvesting process.

Use a clean stainless steel spatula to remove any soil from the reactors. Clean the spatulae with distilled water, rinse with ethanol, then flame it over a Bunsen burner between each reactor harvest to reduce carryover of contaminants, soil constituents, and microorganisms between samples. Allow the spatula to cool to room temperature before inserting it into the next reactor.

Transfer soil samples into clean acid-washed bottles or vials and seal with Teflon™-lined caps. Analyze samples as soon as possible. Harvest each reactor in a consistent manner to ensure proper sampling and sample handling techniques are followed.

To harvest the reactors, remove the flask stopper and retrieve the soil sample with the sterilized stainless-steel spatula. After each soil removal reset the stopper into the flask.

First, remove approximately 3 mL of soil and transfer it into a clean 3-mL Teflon™-lined screw-cap vial. Fill the vial to the top in order to limit the amount of headspace and volatile loss of hydrocarbon. Seal the vial as quickly as possible. Submit this sample for soil TPH analysis.

Next, remove approximately 10 g of soil and transfer it into a preweighed crucible with lid. Place the lid on top of the crucible and immediately record the weight of the sample and crucible with lid. Use these samples for moisture content analyses and subsequently for organic matter analyses.

Remove approximately 5 g of soil from the reactor and place the soil into a 20-mL glass screw-cap scintillation vial. Add 5 mL of Milli-Q grade water and screw the cap back onto the vial. This vial will be used for pH analysis. Finally, transfer the remaining soil into an I-Chem® bottle for nutrient and soil properties analyses.

4.0 ANALYTICAL METHODS

4.1 Petroleum Hydrocarbon Concentrations in Soil Samples

Soil samples are analyzed on a Hewlett-Packard (HP) 5890 GC using a heated purge-and-trap method. The GC is equipped with a 30-m DB-1 wide-bore capillary column connected to a flame ionization detector (FID). The initial oven temperature is held at 20°C with cryogen for 4 minutes, then ramped at 10°C/min to 240°C and held for 4 minutes or until a stable baseline is achieved. Peak elution along with resulting area counts are recorded and stored as computerized files using the Chromperfect® for Windows data acquisition software package. The concentrations of the 20 compounds listed in the calibration mixture are calculated by applying response factors determined from responses from injections of known concentration.

Weigh out approximately 1 g of soil into a test tube and add 5 mL of Milli-Q water. Attach the test tube to the purge-and-trap concentrator fitted with the sterilized soil sparging needle. Purge the sample for 8 minutes at 85°C and collect the purged hydrocarbons on the sorbent trap. Desorb the trapped organics to the GC for compound resolution and quantification.

4.2 Petroleum Hydrocarbon Concentrations in Gas Samples

Gas samples collected during atmospheric exchanges are analyzed for petroleum hydrocarbons. After the three gas samples of each condition are processed for oxygen and carbon dioxide

concentrations, 50 mL of each of the gas samples are pulled by a 200-mL gastight syringe and are injected into one Tedlar™ sampling bag to create a composite sample of each of the three conditions. By creating this composite sample bag at the analytical stage rather than averaging the three values at the data reduction stage, the analysis time of the samples is cut by two-thirds. An HP 5890 GC equipped with a 60-m DB-1 wide-bore capillary column (J & W Scientific) connected to a FID is used to analyze the composite gas samples for petroleum hydrocarbons. An auto sampler attached to the six-port valve injection port equipped with a heated sample loop is used to introduce 2 mL of the sample into the GC. The oven temperature is held at 20°C for 4 minutes, then ramped at 10°C/min up to 180°C and held for 12 minutes. The data are collected by Chromperfect® and the concentrations of the specific hydrocarbons are calculated using Chromperfect® by multiplying the resulting area count for each compound by the response factor. The response factors are calculated by dividing known concentrations of each of the 20 compounds by their respective area counts. The area counts are determined by injecting and analyzing a calibration standard at that concentration.

4.3 Oxygen and Carbon Dioxide Concentrations in Gas Samples

Oxygen and carbon dioxide are measured in the gas collected from each reactor flask during the atmosphere exchanges. When gas samples are collected, analyses for oxygen and carbon dioxide concentrations are performed prior to petroleum hydrocarbon gas analysis. The oxygen concentration data are used to ensure that the oxygen levels do not become rate-limiting and to determine the biodegradation rates over the incubation period. An SRI GC equipped with a CTR-I concentric column (Altech) connected to a thermal conductivity detector (TCD) is used to analyze the gas samples for oxygen and carbon dioxide concentrations. An isothermal method at ambient temperature is used, with helium serving as the carrier gas. A 10-mL sample volume is injected through a multiport valve injector assembly which contains a 2-mL looped system. The concentrations of oxygen and carbon dioxide are calculated using response factors generated from a multipoint calibration from injections of standards of known concentrations.

4.4 Microbial Enumerations in Inoculum

Initial microbial enumerations are conducted on the microbial amendment inoculum to determine the number of cells/g dry soil added. Enumerations of the amendment are conducted in triplicate using a serial dilution and spread-plate method. The procedure for this analysis is as follows:

Step 1. Prepare 0.2M solutions of K_2HPO_4 and KH_2PO_4 in distilled water. Mix the solutions in the ratio 77 parts K_2HPO_4 to 28 parts KH_2PO_4. Adjust the pH to 7.2 using KOH or HCl. Dispense 9-mL aliquots of the potassium phosphate buffer into 30-mL borosilicate glass test tubes for dilution blanks. Cap and autoclave for sterilization. After sterilization, store the tubes in a refrigerator at 10°C until they are ready to be used. Allow the tubes to equilibrate to room temperature before using.

Step 2. Prepare a basal inorganic medium by adding 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.05 g $CaSO_4 \cdot 2H_2O$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 1.0 g $(NH_4)_4SO_4$ into 1.0 L of distilled water and adjust the pH to 7.2 using KOH or HCl. Add 20 g noble agar (purified agar) to the medium and stir with heat until the agar has dissolved. Autoclave for sterilization. After sterilization, dispense 20-mL aliquots into petri dishes. After the medium has solidified in the petri dishes, label the bottom of the petri dishes with the dilution and replicate information.

Step 3. Aseptically transfer 1 mL of prepared amendment (prepared according to the company's directions) into triplicate tubes containing 9 mL of dilution blank. Vortex for 10 seconds. Aseptically transfer 1 mL of this suspension to a dilution blank containing 9 mL of buffer and vortex for 5 seconds. Continue with this dilution process until the desired number of dilutions have been made.

Step 4. Aseptically transfer 0.1 mL of each dilution to the appropriate plate. Spread using a glass spreader that has been sterilized with ethanol and then flamed. Allow the flame on the glass spreader to go out by itself to ensure all ethanol has been burnt off. Also take care to use the glass spreader only after it has cooled to ensure that the microbes are not killed by the heat. Invert the plates and place inside a gas pack. Place an absorbent pad with 1 mL of JP-4 jet fuel into the gas pack and seal the gas pack. Incubate at 25°C until colonies appear and then count the colonies.

4.5 Soil Moisture

Initial and final soil moisture content analyses are conducted to determine whether the soils contain suitable water content to support microbial growth and nutrient transfers, to monitor the net water loss by the end of the incubation period, and to determine a constant that is used in soil dry-weight calculations. A gravimetric method (Gardner, 1965) is used to make the moisture determination. Soil moisture analyses are conducted as follows:

Step 1. Turn on the drying oven and allow the temperature to equilibrate at 105°C.

Step 2. Preweigh crucibles with crucible lid and label each by etching both with an ID number.

Step 3. Weigh out approximately 5 g of soil in triplicate and place in the preweighed, labeled crucibles. Cover the samples with the respective crucible lid.

Step 4. Place covered crucibles containing the soil samples into the drying oven and allow them to dry for 24 hours.

Step 5. Remove the crucibles from the drying oven and place them into a desiccator at room temperature. Allow the crucibles to cool to constant weight at room temperature.

Step 6. Remove the crucibles from the desiccator and record the cumulative weight of the crucible plus the sample after drying.

Step 7. Calculate the percent soil moisture content with the following formula:

$$\% \text{ moisture content} = \frac{(\text{weight of soil})_{wet} - (\text{weight of soil})_{dry}}{(\text{weight of soil}_{dry} - \text{tare})} \times 100 \qquad (1)$$

4.6 Organic Matter Content in Soil Samples

Organic matter is measured in initial and final soil samples to characterize the soil samples and to monitor carbon loss due to biodegradation. The analysis is conducted on the samples used to determine the soil moisture content as follows.

Step 1. Turn on the muffle furnace and equilibrate temperature at 550°C.

Step 2. After the final cumulative weights are determined in Step 6 of the soil moisture content procedure, place the covered crucibles with sample into the muffle furnace and close the muffle furnace door. Allow the samples to combust at 550°C for 1 hour.

Step 3. After 1 hour remove the crucibles from the muffle furnace and immediately place the dishes into a desiccator. Allow the crucibles to cool to constant weight at room temperature.

Step 4. Remove each crucible from the desiccator and record the cumulative weight of the dish plus the sample after muffling.

Step 5. Calculate the soil organic matter content using the following formula:

$$\text{organic matter content (\%)} = \frac{(\text{weight of soil})_{dry} - (\text{weight of soil})_{cmbstd}}{(\text{weight of soil})_{dry}} \times 100 \qquad (2)$$

4.7 Nutrient Concentrations in Soil Samples

Initial and final soil samples are analyzed for calcium, magnesium, potassium, phosphorus, and nitrogen. The following is the detailed procedure used to measure each analyte:

4.7.1 Calcium, Magnesium, and Potassium. Follow the procedure below to measure the calcium, magnesium and potassium concentrations in soil samples. The volumes may be reduced depending on soil availability.

Prepare the following reagents:

1. Extracting Solution: Dispense approximately 100 L of water into a 30-gal reagent tank calibrated to 115 L. Add 6,560 mL of concentrated acetic acid. Then add 7,900 mL of concentrated ammonium hydroxide. Allow the solution to cool and adjust the pH to 7.0 with acetic acid or ammonium hydroxide. Bring the volume to 115 L and mix. The pH of this solution should be monitored.

2. Stock Standards: Ca = 10,000 ppm; Mg = 1,000 ppm; K = 1,000 ppm.

3. Working Standards:*

   a. Ca = 2,000 ppm and Mg = 300 ppm: Add 200 mL of Ca Stock Standard and 300 mL of Mg Stock Standard to a 1,000-mL volumetric flash, and dilute to volume with Extracting Solution.

   b. Ca = 1,000 ppm and Mg = 150 ppm: Dilute 250 mL of Standard A to 1,000 mL with Extracting Solution.

   c. Ca = 500 ppm and Mg = 75 ppm: Dilute 250 mL of Standard A to 1,000 mL with Extracting Solution.

   d. K = 25 ppm: Add 25 mL of the K Stock Standard to a 1-L volumetric flash and dilute to volume with deionized water.

   e. K = 50 ppm: Dilute 50 mL of the K Stock Standard to 1,000 mL with deionized water.

   f. K = 100 ppm: Dilute 100 mL of the K Stock Standard to 1,000 mL with Extracting Solution.

*Note that the potassium standards have been made in water rather than extracting solution.

Use the following procedure to prepare the sample and analyze for the listed nutrients.

Step 1. Measure 5 g of soil into a 20-dram vial using a standard NCR-13 scoop.

Step 2. Dispense 25 mL of Extracting Solution. Seal and shake for 15 minutes at 180 rpm.

Step 3. Filter through Whatman #2 paper and filter if the extract is cloudy.

Step 4. Determine the Ca, Mg, and K levels by atomic absorption.* A blank must be carried through with each set. (ppm in the soil = 5 × ppm in the extract.)

4.7.2 Phosphorous. Use the following procedure to measure phosphorus in soils. First prepare the reagents listed below.

1. Weak Bray Solution: Weigh 127.7 g of ammonium fluoride into a 30-gal Nalgene tank marked for 115 L. Add approximately 30 L of deionized water. Add 240 mL of concentrated HCl. Dilute to 115 L with deionized water and mix well with an electric mixer.

2. Strong Bray Solution: Weigh 127.7 g of ammonium fluoride into a 30-gal Nalgene tank marked for 115 L. Add about 30 L of deionized water. Add 958 mL of concentrated HCl and dilute to 115 L with deionized water, mixing with an electric mixer.

3. P_2 Solution (Molybdate): Weigh 100 g of ammonium molybdate crystals into a 1,000-mL graduated cylinder. Add deionized water to 850 mL. Mix until the solution is homogeneous. Add this solution to the P_2 reagent bottle, and add 1,700 mL of concentrated HCl with mixing.

4. P_3 Solution (Color Developing Reagent): Place 50 g of sodium sulfite and 25 g of 1-amino-2-naphthol-4-sulfonic acid in a large mortar and grind thoroughly until the color is evenly distributed. Add 1,463 mL of sodium bisulfite. Grind and mix again until the color is uniform. Dry overnight at 105°C. Prepare the solution by dissolving 27.0 g of the dry mixture in distilled water, and dilute to 1,000 mL. (A warm water bath may facilitate dissolution.) Make this solution once per week as it has limited stability.

5. Stock Phosphorus Solution (100 ppm): Dissolve 0.4394 g of dried KH_2PO_4 in approximately 250 mL of deionized water in a 1,000-mL volumetric flash.** Dilute to volume and mix well.

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*The source recommends use of flame emission spectroscopy for determination of K.

***Methods of Soil Analysis: Part 2 – Chemical and Microbiological Properties*, 2nd ed. (ASA, 1982), recommends the addition of five drops of toluene to the phosphorus stock standard to diminish the possibility of microbial activity.

6. Working Phosphorus Standards (1 ppm, 5 ppm, 10 ppm): Accurately pipette 10 mL of the stock standard into a 1,000-mL volumetric flash. Dilute to volume, and mix well. Follow this same procedure using 50 mL and 100 mL to make the 5-ppm and 10-ppm working standards. Do not add the aliquot of stock standard after bringing to volume. The final volume must be 1,000 mL.

Use the following procedure.

Step 1. Weak Bray Extraction:

a. Measure 1 g of soil into the phosphorus extraction cup.

b. Add 7 mL of Weak Bray Solution to the cup and shake for 1 minute.

c. Filter through a medium retention filter paper into small phosphorus vials. Develop the color and read on the Gilford as outlined below.

Step 2. Strong Bray Extraction:

a. Measure 1 g of soil into a phosphorus extraction cup.

b. Add 7 mL of Strong Bray Solution to the cup and shake for 1 minute.

c. Filter the extract through a medium retention filter into small phosphorus vials.

Step 3. Color Development:

a. Refilter any extract that is cloudy. All extracts must be clear.

b. Set up the standard curve by adding 4.5 mL of deionized water, 4.5 mL of the 1- ppm standard, 4.5 mL of the 5-ppm standard, and 4.5 mL of the 10-ppm standard into small phosphorus vials, and proceed with the color development.

c. Add 0.3 mL of P_2 solution to each standard, blank, and soil extract using the Oxford Adjustable Dispenser.

d. Add 0.3 mL of Pb_3 to each standard, blank, and soil extract using another Oxford Adjustable Dispenser.

e. Let stand for 30 minutes before reading to ensure complete color development.

f. Set up a standard curve on the Gilford Spectrophotometer at 660 nm in the direct concentration mode at the following settings:

Blank = 0000
1 ppm = 0070
5 ppm = 0350
10 ppm = 0700

Notes:

(1) The dispensers used for the weak and strong brays must be checked daily to ensure that the correct volume (7 mL) is being dispensed. They should also be checked while in use.

(2) The volumes of P_2 and Pb_3 solutions being dispensed should also be checked daily and throughout the procedure.

(3) The P_2 (Molybdate) Solution is made from concentrated HCl. Use the eye protection provided and avoid contact with the skin. Keep the area well ventilated.

4.7.3 Total Nitrogen. Use the following procedure to measure total nitrogen in the soil samples. First prepare the following reagents.

1. Potassium Sulfate-Catalyst Mixture: Prepare a mixture of 200 g of potassium sulfate, 20 g of cupric sulfate pentahydrate, and 2 g of Se. Powder the reagents separately, and grind the mixture in a mortar to powder the cake that forms during mixing.

2. Concentrated and Standardized 0.1N Sulfuric Acid.

3. Sodium Hydroxide Solution ($\simeq$10N): Place 3.2 kg of reagent-grade NaOH in a heavy-walled 10-L Pyrex™ bottle marked up to 8 L. Add 4 L of carbon dioxide-free water, and swirl the bottle until the alkali is dissolved. Cool the solution with a rubber stopper in the neck of the container, and bring it to 8 L with carbon dioxide-free water. Swirl vigorously, and store the bottle with some arrangement that allows the alkali to be stored and dispensed with protection from CO_2.

4. Mixed Indicator Solution: Dissolve 0.099 g of Bromcresol Green and 0.066 g of Methyl Red in 100 mL of ethanol. Now add 0.1N NaOH cautiously until the solution assumes a reddish-purple tint (pH $\simeq$5). Make the solution to 4 L by adding water. Mix thoroughly.

5. Boric Acid-Indicator Solution: Place 80 g of boric acid in a 5-L flask marked to indicate a volume of 4 L. Add 3,800 mL of water and heat and swirl the flask until the boric acid is dissolved. Cool the solution and add 80 mL of the mixed indicator solution.

Use the following procedure to prepare and analyze the soils.

Step 1. Place a sample expected to contain about 1 mg of nitrogen in a micro-kjeldahl digestion flask, add 1.1 g of the potassium sulfate-catalyst mixture and 3 mL of concentrated sulfuric acid, and heat the flask cautiously on the digestion stand.

Step 2. When frothing has ceased, increase the heat until the digest clears, and then adjust the heat so that the reflux line for the sulfuric acid appears about one-third of the way up the neck of the digestion flask. Continue the digestion for 5 hours.*

Step 3. Allow the flash to cool, and slowly add 20 mL of water. Swirl to suspend solids, and transfer the contents to the distillation chamber of the Hoskins apparatus via the funnel. Rinse the kjeldahl flash 3 times with a total of about 9 mL of water and add the rinsings to the digestion flash. Bring the solution level up to a mark made previously to indicate a volume of 50 mL, and close the stopcock connecting the funnel and distillation chamber.

Step 4. Add 5 mL of boric acid-indicator solution to a 50-mL Erlenmeyer flash previously marked to indicate a volume of 35 mL, and place the flash under the condenser so that the end of the condenser is about 4 cm above the solution.

4.8 Soil pH

The soil pH is determined before and after incubation. The initial soil pH values are determined for each experimental condition before incubation and soil pH is determined after the incubation period for each reactor. The pH of the soils is determined as follows:

Step 1. Calibrate the pH-meter using a dual-point calibration with pH 4.00 and pH 7.00 standards. Check the calibration slope to make sure the probe is in good working order.

Step 2. Add approximately 5 g of soil to 5 mL of Milli-Q water in a prelabeled 20-mL screw-cap glass scintillation vial.

Step 3. Screw the cap onto the vial and allow the slurry to mix on a bench-scale shake table at 200 rpm for 4 days. This will provide ample time for equilibration of the soil/water matrix.

Step 4. After the 4-day equilibration period, remove the cap from the vial and insert a small magnetic stir bar. Place the vial with stir bar onto a stir plate and allow to mix for 15 seconds.

Step 5. Place the pH electrode into the soil/water suspension and record the displayed pH value.

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*The ammonia-nitrogen produced by digestion for 2 hours is rarely less than 98% of that formed in the recommended 5 hours. According to the source, even a 1-hour digestion period is adequate for routine soil analyses not requiring high degrees of accuracy.

4.9 Physical Properties of Soil Samples

Physical soil characteristics including such as particle size distribution and CEC are important parameters that affect microbial activity and will be measured on all initial soils samples. In addition the data is used to extrapolate the results from an amendment test to soils having similar characteristics from other sites.

4.9.1 Particle Size Distribution and Textural Analyses. Particle size distribution analysis is conducted for the initial soils. Soil particle size and percent sand, silt, and clay are determined using timed hydrometer readings in a modified Stoke's law equation. Soil texture is determined by comparing particle size with the U.S. Department of Agriculture (USDA) soil texture triangle. A detailed description of the method for particle size distribution analysis follows:

Prepare or obtain the following item and reagents:

1. Sodium Hexametaphosphate Solution: Dissolve 900 g of sodium hexametaphosphate in 18 L of distilled water.

2. Isopropyl Alcohol.

3. Hydrometer: ASTM No. 152H with Bouyoucos scale in g/L, T=67°F.

Use the following procedure to determine particle size:

1. Tag a plastic cup with a sample number. Then tare the plastic cup and weigh out 50 g of sample (25 or 10 g may be used if there is not enough sample; 100 g should be used for sandy soils). Record the weights.

2. Transfer the number tag to a blender cup. Add the sample to 20 mL of sodium hexametaphosphate solution. Dilute to 100 mL with distilled water adjusted to 67°F. Blend for 5 minutes.

3. Start the stopwatch and adjust the sink temperature to 67°F.

4. Transfer the number tag to a 1,000-mL cylinder, and wash the sample into the cylinder with distilled water, bringing the total volume to 1,000 mL.

5. Stopper the cylinder and thoroughly mix the contents by inverting it several times while shaking. Add one drop of isopropyl alcohol if the mixture is considerably foamy.

6. Set the cylinder on the table and record the starting time. Place the hydrometer and thermometer into the suspension, and read both instruments exactly 40 seconds after the starting time.

7. Let the cylinder stand for 2 hours. Replace the hydrometer into the suspension and again read the hydrometer at water level. Record the reading and the temperature. Rinse the cylinder.

8. Take the results to the computer for analysis of percent sand, percent silt, and percent clay, and use these values to name the soil texture according to the USDA soil texture triangle diagram supplied with this method.

Make the following hydrometer adjustments and calculations:

1. Hydrometer Correction: Because the hydrometers are calibrated at 67°F, the temperature dependency of Stoke's law must be adjusted for variations in the experimental temperatures. According to L.D. Baver, *Soil Physics*, (3rd ed.), "the (terminal) velocity of fall at 30°C is about 12 percent faster than at 25°C." The Iowa State University soil science lab manual suggests that this relationship between temperature and terminal velocity yields the following hydrometer temperature dependency:

> "For each degree above the calibration temperature, add 0.2 g of soil to the reading to get the corrected hydrometer reading. For each degree below the calibration temperature, subtract 0.2 g of soil from the reading."

Therefore, to get the actual hydrometer reading, incorporate the temperature correction as stated above, and subtract the following experimentally determined blank readings:*

Temp.	Subtract
66-68°F	0
61-65°F	1
55-60°F	2

2. Percent Sand:

For 50-g samples:

$$\% \text{ Sand} = 100 - (\text{Corrected 40-second hydrometer reading}) \times 2.0$$

For 25-g samples:

$$\% \text{ Sand} = 100 - (\text{Corrected 40-second hydrometer reading}) \times 4.0$$

For 100-g samples:

$$\% \text{ Sand} = 100 - (\text{Corrected 40-second hydrometer reading})$$

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*This linear temperature correction is probably only valid over a small range of temperatures (i.e., ±3°F from the calibration temperature).

3. Percent Clay:

For 50-g samples:

$$\% \text{ Clay} = (\text{Corrected 2-hour hydrometer reading}) \times 2.0$$

For 25-g samples:

$$\% \text{ Clay} = (\text{Corrected 2-hour hydrometer reading}) \times 4.0$$

For 100-g samples:

$$\% \text{ Clay} = \text{Corrected 2-hour hydrometer reading}$$

4. Percent Silt:

$$\% \text{ Silt} = 100 - (\% \text{ Sand} + \% \text{ Clay})$$

4.9.2 Cation Exchange Capacity. Initial soil samples are analyzed for their cation exchange capacity (CEC) by using an ammonium saturation method. Soil is leached with an excess of neutral 1N ammonium acetate solution to remove the exchangeable cations and to saturate the exchange material with ammonium ions. After removal of excess ammonium present in the soil as the acetate, the exchangeable ammonium is determined by displacement with NaCl and distillation. The following is a detailed description of this method.

To prepare the reagents:

1. Saturating Solution (Ammonium Acetate, 1N): Dilute 114 mL of glacial acetic acid (99.5%) with distilled water to a volume of approximately 1 L. Add 138 mL of concentrated ammonium hydroxide and dilute to approximately 1,980 mL. Adjust to pH 7.0 with ammonium hydroxide or acetic acid. Dilute to 2 L with distilled water.

2. Isopropyl Alcohol (99%).

3. Ammonium Chloride (1N): Adjust to pH 7.0 with ammonium hydroxide.

4. Ammonium Chloride (0.25N): Adjust to pH 7.0 with ammonium hydroxide.

5. Ammonium Oxalate [$(NH_4)_2C_2O_4 \cdot H_2O$, 10%].

6. Dilute Ammonium Hydroxide: Add 1 volume of concentrated ammonium hydroxide to an equal volume of distilled water.

7. Silver Nitrate (0.10N).

8. Sodium Chloride (acidified): Prepare a 10% aqueous solution of NaCl (ammonia-free) by dissolving 100 g of NaCl in 990 mL of distilled water. Add 0.5 mL of HCl (or enough HCl to bring the solution to a pH of approximately 5.3).

9. Sodium Hydroxide (1N).

10. Boric Acid (2%).

11. Standardized Sulfuric Acid (0.1N).

12. Bromcresol Green-Methyl Red Mixed Indicator Solution: Tritate 0.1 g of Bromcresol Green with 2 mL of 0.1N NaOH in an agate mortar, and add 95% ethanol to obtain a volume of 100 mL. Tritate 0.1 g of Methyl Red with a few mL of 95% ethanol in an agate mortar. Add 3 mL of 0.1N NaOH, and dilute the solution to a volume of 100 mL with 95% ethanol. Mix 75.0 mL of the Bromcresol Green solution with 25.0 mL of the Methyl Red solution. Dilute to 200 mL with 95% ethanol.

Use the following procedure to determine the CEC of the soil:

1. Place 10 g* of air-dried soil in a 500-mL Erlenmeyer flask and add 250 mL of neutral, 1N ammonium acetate solution.

2. Shake the flask thoroughly and allow it to stand overnight.

3. Filter the soil by light suction using a 55-mL Buchner funnel. Do not allow the soil to dry or crack.

4. Leach the soil with the neutral ammonium acetate until the solution is negative on the calcium test.

   Calcium Test:

   a. Place 10 mL of the leachate in a test tube and add a few drops of 1N ammonium chloride.

   b. Add also a few drops of 10% ammonium oxalate and dilute ammonium hydroxide.

   c. Heat to near boiling.

   d. Calcium is indicated by turbidity or white precipitate.

5. When the calcium test is negative, save the leachate for the exchangeable base determination, or discard.

6. Leach the soil four times with neutral 1N ammonium chloride and once with 0.25 ammonium chloride.

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*Use 25.0 g of sample if the CEC is expected to be very low (3 to 5 m_{eq} per 100 g).

7. Wash out the electrolyte with 150 to 200 mL of 99% isopropyl alcohol until the test for chloride in the leachate (using 0.1N silver nitrate) is negligible.

8. Leach the ammonium-saturated soil with 10% NaCl (acidified) by small portions until 225 mL have passed through the sample. Allow each portion to pass through before adding the next.

9. Transfer the leachate quantitatively to an 800-mL kjeldahl flask. Add 25 mL of 1N NaOH and distill 60 mL of the solution into 50 mL of 2% boric acid.

10. Add 10 drops of the Bromcresol Green-Methyl Red mixed indicator solution and titrate with standardized 0.1N sulfuric acid.*

11. Run blanks on the reagents.

12. Correct the titration figure for the blanks, and calculate the m_{eq} of ammonium in 100 g of soil.

Use the following formula to calculate the CEC:

Cation Exchange Capacity (CEC) = m_{eq} of NH_4 per 100 g of sample (m_{eq}/100 g).

5.0 DATA ANALYSIS AND AMENDMENT EVALUATION

The data produced from the analyses conducted under this protocol are used to evaluate the performance of the amendment product against the degradation performance of the indigenous microorganisms without the addition of the amendment. Analyzing the data will allow the determination of any potential enhancements that the amendment provides for a given soil and soil type. The data are analyzed as follows.

5.1 Soil Characteristic Data

The soil characteristic data from the pH, particle size distribution, CEC, organic matter, and alkalinity analyses are used to extrapolate the results from an amendment test to soils having similar characteristics from other sites, to screen potential candidates for further testing for application at that site.

5.2 Microbial Enumeration Data

*The color change for this titration is from bluish-green to bluish-purple.

The data from the enumeration of the microorganisms in the inoculum are used to verify the presence of viable microorganisms that are capable of degrading hydrocarbon. The data also are used to determine the rate at which live microbes are added to the soil. These data can be useful for evaluating rates between amendments on a organism-dosing basis. Amendments that show little or no growth during the enumeration analysis and that do not provide any enhanced degradation are deemed ineffective and are not recommended as candidates for use at Air Force sites.

5.3 Nutrient Data

The nutrient data are used to evaluate the background nutrient levels before amendment addition and the contribution to the nutrient pool from the amendment. The data provide information that is useful for evaluating enhancements observed in the condition containing the sterilized amendment that result from additional nutrients.

5.4 Oxygen Utilization and Carbon Dioxide Production Data

Respiratory data have been used to determine biodegradation rates in laboratory studies and in the field with applications such as bioventing. For the test described in this protocol, the data are used primarily for maintaining aerobic conditions and to indicate the rate of microbial activity in each reactor. Because the amendments often contain biodegradable components, respiration in the reactors could be the result of the degradation of these components and not the contaminant. Because of this, biodegradation rates based on either oxygen utilization or carbon dioxide production may overestimate the hydrocarbon degradation rate.

5.5 Petroleum Hydrocarbons in Soil Gas

The hydrocarbon data from the analysis of the gas collected during atmosphere exchanging are used to determine the mass of hydrocarbon removed from the soil through volatilization. The concentration data obtained from the GC analysis or the composited sample are converted to a mass of hydrocarbon removed by multiplying the concentration value by the volume of gas exchanged (300 mL). The cumulative mass is subtracted from the mass initially present in the soil to determine the amount of hydrocarbon available for biodegradation as described below.

5.6 Petroleum Hydrocarbons in Soil Samples

These are the most critical data with regards to evaluating any enhanced biodegradation due to the addition of the amendment. It is more appropriate to analyze the amendments on a mass-removed basis rather than on a reduction-in-concentration basis. To do this, the concentration data obtained from the GC analysis of the triplicate flasks are averaged and then converted to a mass of hydrocarbon present in the reactor. This is accomplished by multiplying the concentration data by the mass of soil (dry-weight basis) in each reactor.

To calculate the mass of hydrocarbon available for biodegradation, the mass of hydrocarbon removed during atmosphere exchanging is subtracted from the initial mass present in the soil. The mass remaining at the end of the incubation period is subtracted from the resulting initial values to determine the amount of hydrocarbon that was biodegraded in each reactor under each experimental condition.

6.0 REPORTING

The results from the tests described in this protocol are submitted in an evaluation report. The report will contain an introduction section that includes the microbial amendment identification and any pertinent vendor information. An experimental methods section is included that contains descriptions of the amendment preparation and usage procedures. In addition, any modifications made to the protocol described above are included along with an explanation of the need for that modification. The results section contains the results from the hydrocarbon analysis and presents the data in tabular form. The results from the remaining analyses are provided in an appendix. The discussion section focuses on the evaluation and interpretation of the data with emphasis on the performance of the amendment based on the hydrocarbon degradation. The final conclusion section contains the final evaluation of the amendment product. The report is submitted within 30 days following completion of the tests described in Section 4.0.

7.0 REFERENCE

Gardner, W.H. 1965. "Water Content." In C.A. Black, D.D. Evans, J.L. White, L.E. Ensminger, and F.E. Clark (Eds.), *Methods of Soil Analysis, Part 1 - Physical and Mineralogical Properties*, Including Statistics of Measurement and Sampling, American Society of Agronomy, Inc., Madison, WI. pp. 82-127.